Sperm-borne small RNA regulate -tubulin acetylation and epigenetic modification of early bovine somatic cell nuclear transfer embryos

Sperm-borne small RNA regulate α-tubulin acetylation and epigenetic modification of early bovine somatic cell nuclear transfer embryos

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Abstract

Accumulated evidences indicate that sperm-borne small RNA plays a crucial role in embryonic development, especially the absence of the sperm-borne small RNA might be a major cause of the abnormal development of cloned embryos. In this study, we found that sperm-borne small RNA can affect abnormal pronuclear-like structures, postpone the timing of first embryo cleavage, and enhance developmental competence of bovine somatic cell nuclear transfer (SCNT) embryos. In addition, the supplementation of sperm-borne small RNA can significantly increase live birth rates and decrease the birth weights of cloned offspring. To investigate the underlying mechanisms, the levels of α-tubulin K40 acetylation (Ac α-tubulin K40) and histone H3 lysine 9 trimethylation (H3K9me3) during early embryo development were investigated in SCNT embryos with sperm-borne small RNA supplementation (termed as T-NT), compared to those normal SCNT embryos and embryos obtained from standard *in vitro* fertilization (IVF). The results showed that sperm-borne small RNA can significantly decrease the H3K9me3 levels at the pronuclear and two-cell stages, while significantly increase Ac α-tubulin K40 levels at anaphase and telophase of bovine SCNT embryos during the first cleavage. Collectively, our study for the first time demonstrates that sperm-borne small RNA plays a crucial role in the developmental competence of SCNT embryos by regulating H3K9me3 and Ac α-tubulin K40. Further studies will be required to determine how sperm small RNA regulate the H3K9me3 and Acα-tubulin K40. Our study suggests that the supplementation of sperm-borne small RNA is a potential application to improve the cloning efficiency.

Keywords: sperm-borne small RNA; embryonic development; α-tubulin K40 acetylation; H3K9me3; the first cleavage; cloning efficiency

Introduction

Sperm was thought only to transfer the paternal haploid genome to the oocyte and then embryo activation occurs upon fertilization. Most nutrient substances and development regulators are provided by the oocyte, in particular the regulatory factors involved in the embryonic development and epigenetic reprogramming (Maki, Suetsugu-Maki et al. 2010). However, in recent years, increasing evidences suggest mature spermatozoon contain coding RNA and non-coding RNA including miRNA (microRNA), piRNA (Piwi-interacting RNA), endo-siRNA (endogenous small interfering RNA), and tRNA (transfer RNA) (Yan, Morozumi et al. 2008, Song, Hennig et al. 2011, Peng, Shi et al. 2012, Jodar, Selvaraju et al. 2013). Some studies have shown that the sperm-borne RNA delivered plays an important role in embryonic development following fertilization (Ostermeier, Miller et al. 2004, Amanai, Brahmajosyula et al. 2006, Yan, Morozumi et al. 2008). In particular, some sperm-borne non-coding RNAs act as epigenetic modifiers and play an important role in epigenetic reprograming and transgenerational epigenetics (Krawetz 2005, Rassoulzadegan, Grandjean et al. 2006, Sato, Tsuchiya et al. 2011, Wang, Gao et al. 2017). For example, miRNA-34c has been identified to be important during the first cleavage of embryos (Liu, Pang et al. 2012). In our previous studies, we found that miRNA-449b, as a sperm-specific one, plays a crucial role in nuclear epigenetic reprogramming of bovine somatic cell nuclear transfer (SCNT) embryos (Wang, Gao et al. 2017). Besides, the birth rates of ICSI embryos derived from spermatozoa with aberrant miRNA and endo-siRNA profiles are increased by a supplementation with wild-type sperm RNA(Yuan, Schuster et al. 2016). The deletion of sperm RNA resulted in reduced body weights of the F1 generation (Guo, Chao et al. 2017). However, the underlying mechanisms on the regulation of sperm-borne small RNA in the embryonic development are remained unclear.

SCNT has become a powerful technique and shown great promise in various field

including basic research, agricultural production and biomedical research (Matoba and Zhang 2018). However, the low efficiency of SCNT and a high proportion of abnormal offspring limit its practical application. Taking bovine as an example, large offspring syndrome (LOS) is the most common abnormal development, resulting in a severe loss of the cloned offspring in perinatal period(Smith, Suzuki et al. 2012). Numerous studies have reported that the incomplete epigenetic reprogramming of nuclear donor cells is a main cause for low embryonic development efficiency and abnormality of cloned offspring (Akagi, Geshi et al. 2013, Tao, Zhang et al. 2017). The common epigenetic reprogramming occurring during early embryonic development includes DNA methylation, histone modification, and chromatin remodeling(Betthauser, Pfister-Genskow et al. 2006). Though various epigenetic reprogramming errors have been observed in SCNT embryos (Yan, Zhou et al. 2010, Matoba, Liu et al. 2014), recent studies indicate that the reprogramming of histone H3 lysine 9 trimethylation (H3K9me3) is an important epigenetic obstacle for efficient SCNT reprogramming. The reduction of H3K9me3 level in nuclear donor cells or early embryos significantly improve nuclear epigenetic reprogramming and embryonic developmental competence (Matoba, Liu et al. 2014, Chung, Matoba et al. 2015). Therefore, H3K9me3 level is now commonly used as an important marker for evaluating nuclear reprogramming efficiency in SCNT embryos.

The cytoskeleton plays an important role in cell movement, cytokinesis, and organization of the organelles within cells (Chakrabarty, Das et al. 2011, Gao, Li et al. 2016). Recent studies indicate that there are various epigenetic modifications on the amino acid residues of the cytoskeleton, with a similar pattern to those on chromosomes. In particular, the acetylation of α-tubulin is an important and well-studied posttranscriptional modification of cytoskeleton (Nakagawa, Suzuki et al. 2013). Altering the level of acetylated α-tubulin has a significant effect on the developmental kinetic and competence of early embryos by

affecting the stability of microtubules (Matsubara, Lee et al. 2013). Comparing SCNT and IVF early embryos, significant differences in cytoskeletal organization and posttranscriptional modification of cytoskeleton were observed, which might be responsible for the abnormal development of SCNT embryos, in particular abnormal pronuclear structures, faster development kinetics prior to 8-cell stage, and higher ratio of blastomere fragments (Shin, Park et al. 2002, Zhu, Jiang et al. 2007, Matoba and Zhang 2018). Therefore, correcting epigenetic modification abnormalities of the cytoskeleton is a potentially promising strategy alternative for improving cloning efficiency.

In this study, we first observed that the sperm-borne small RNA plays a crucial role in the development competence of SCNT embryos in bovine, in particular pronuclear formation, developmental kinetics, cleavage, blastocyst formation rate, apoptotic index, Regarding the inner cell mass (ICM)/tropho ectoderm (TE) ratio, developmental competence in vivo, birth rate, and healthy status of cloning offspring. In addition, the alteration of epigenetic reprogramming in chromosomes and cytoskeleton was evaluated in early embryos to partially uncover the underlying mechanisms.

Material and Methods

All chemicals and reagents were purchased from Sigma (St Louis, MO, USA) unless otherwise stated. Disposable sterile plasticware was purchased from Nunclon (Roskilde, Denmark). All experimental procedures and methods were approved by the Animal Care and Use Committee of Northwest A&F University and performed in accordance with animal welfare and ethics guidelines.

Oocyte collection and *in vitro* maturation (IVM)

Oocyte collection and *in vitro* maturation were carried out as described previously (Wang, Tang et al. 2011). Briefly, bovine cumulus–oocyte complexes (COCs) were aspirated from 2

to 8 mm antral follicles of slaughter-house ovaries before three washes in phosphate-buffered saline (PBS) supplemented with 5% (v/v) fetal bovine serum (FBS). Oocytes with a multilayered compact cumulus and a uniformly dark cytoplasm were selected and picked up under a microscope. After washing three times with PBS, oocytes cultured *in vitro* in bicarbonate-buffered tissue culture medium 199 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) FBS, 1 μg/mL 17ß-estradiol, and 0.075 U/mL human menopausal gonadotropin for 20 h at 38.5°C in an atmosphere of 5% CO_2 in air.

Somatic cell nuclear transfer

The procedures for generating SCNT embryos were conducted as described previously (Qu, Qing et al. 2017). After maturation, COCs were treated with 0.1% bovine testicular hyaluronidase in PBS to remove the cumulus cells from the oocytes. Prior to SCNT, metaphase II (MII) oocytes with first polar bodies were selected and stained with 10 μg/mL Hoechst 33342 for 10 min. Enucleation was performed using a 20 μm glass pipette by aspirating the first polar body and a small amount of the surrounding cytoplasm in microdrops of PBS supplemented with 7.5 μg/mL cytochalasin B (CB) and 10% FBS. The extracted cytoplasm was examined under ultraviolet illumination in another microdrop to achieve successful enucleation. The disaggregated donor somatic cell, derived from a 1-week-old Holstein heifer, was transferred to the previtelline space of an enucleated oocyte. The oocyte–cell couplet was fused with a double electrical pulse of 35 V for 10 μs in microdrops of Zimmermann's fusion medium. Successfully reconstructed embryos were incubated in mSOFaa (modified synthetic oviductal fluid containing Essential amino acid and non-essential amino acid) containing 5 μg/mL cytochalasin B for 2 h, activated in 5 μM ionomycin for 4 min, and exposed to 1.9 mM dimethynopyridine in mSOF for 4 h. After activation, the reconstructed embryos were cultured in 50 μL drops of mSOFaa medium supplemented with 8 mg/mL BSA under a humidified atmosphere of 5% CO_2 in air at 38.5°C.

Cleavage and blastocyst formation ratios were determined at 24 h, 48 h, 6 d, and 7 d after activation.

In vitro fertilization (IVF)

Mature sperm were isolated from the cauda epididymis of male bovines as described previously(Du, Wang et al. 2014), and prepared for IVF. Briefly, fresh semen was placed at the bottom of a 15 mL tube with 5 mL of Brackett and Oliphant (BO) medium supplemented with 6 mg/mL BSA and 20 μg/mL heparin, and then incubated for 30 min at 38.5°C under 5% CO_2 in air. The sample was centrifuged twice at 500 g for 5 min, and the supernatant was removed. The spermatozoa were used for *in vitro* fertilization or RNA isolation. IVF was carried out as described previously (Du, Wang et al. 2014). Briefly, 10^5 spermatozoa in a 50 μL microdrop of BO-IVF medium (IVF Bioscience, Falmouth, United Kingdom) were added to 20–25 COCs under mineral oil. After 16 h of insemination, zygotes from which cumulus cells had been removed and redundant spermatozoa were cultured in mSOFaa containing 8 mg/mL BSA at 38.5°C under 5% CO_2 in air.

Small RNA isolation, reverse-transcription PCR (RT-PCR), and quantitative real-time PCR (qPCR)

Sperm was firstly isolated and purified by a 'swimming-up' method as described above (Du, Wang et al. 2014). QIAzol Lysis Reagent was subsequently added to lyse the sperm and then chloroform was added to the sample. Oocytes and embryos cultured were lysed in the lysis/binding solution as described previously(Qu, Zhao et al. 2018). Then the homogenate from each sample was processed in small RNA extraction using the miRNeasy Micro Kit (Cat. #217084 ; QIAGEN, Duesseldorf, Germany) following the manufacturer's instructions. Briefly, the homogenate was vigorously shaken, incubated at RT (room temperature), and centrifuged at 12000 g for 15 min at 4°C. After that, one volume of 70% ethanol was added

in and the volume was mixed thoroughly. The reaction was transferred to an RNeasy MinElute spin column, followed by an elution step. Approximately 2/3 total volume of 100% ethanol was added in the flow-through, and the mixture was then transferred to a new RNeasy MinElute spin column. This was followed by elution with RWT buffer and RPE buffer, respectively. Then the final elution step with 80% ethanol was performed on the column, followed by adding RNase-free water on spin column membrane, such that the flow-through containing small RNA fractions was harvested. The small RNA quality was assessed using Nanodrop as described previously (Ma, Zhang et al. 2018). The sperm small RNA was then used for injecting into embryos after SCNT, while the small RNA isolated from oocytes or embryos were used for RT-PCR and qPCR.

The procedure of RT-PCR and qPCR was followed as our previous report (Ma, Zhang et al. 2018). The small RNA-enriched fraction was reverse-transcribed using a Mir-X miRNAs First-Strand Synthesis Kit (Cat. # 638315, Takara, Tokyo, Japan). In a simple, single-tube reaction, the small RNA molecules are polyadenylated and reverse transcribed using poly(A) polymerase. To estimate the small RNA yield, two previously reported sperm-borne small RNA, miR-34c(Wang, Wang et al. 2014) and miR-449b(Wang, Gao et al. 2017), were quantified using StepOne Plus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). U6, a most commonly used internal control, was used for data normalization of the small RNA. The reverse primers of miR-34c, miR-449b and U6 for reverse-transcription were provided by the Mir-X miRNA First-Strand Synthesis Kit. Primers of miR-34c, miR-449b and U6 for qPCR contain a forward primer and a universal reverse primer, and the universal reverse primer was provided by Mir-X™ miRNA qRT-PCR TB Green™ Kit (Takara, Cat. # 638316). The sequences of forward primers used in this study are showed as follows: miR-34c-F (5′-GGGAGGCAGTGTAGTTAGCTGATT-3′);miR-449b-F(5′-GGGAGGCAGTGTATTGTTAGCTG-3′) ; U6-F (5 ′CGCTTCGGCAGCACATATACTA-

3′).

Injection of sperm small RNA into embryo after SCNT

The concentration of sperm small RNA was adjusted to 2 ng/μL as described previously(Yuan, Schuster et al. 2016). 2 μL of the diluted small RNA solution was loaded into a microinjection needle (Eppendorf, Hamburg, Germany). The reconstructed embryos were injected with 2 pL of miRNAs solution in HEPES-CZB medium 1 h after their activation. Successful injected embryos had distinct cytoplasm and slightly swelled. They were then transferred into pre-balanced mSOF medium and cultured at 38.5°C in an atmosphere of 5% CO_2 in air.

Immunofluorescence and confocal microscopy

Immunofluorescence staining was performed as described previously(Lu, Zhang et al. 2019). Briefly, embryos were washed three times and then fixed with 4% paraformaldehyde in PBS for 30 min at RT. They were then permeabilized with 1% Triton X-100 in PBS, and incubated in blocking solution (1% bovine serum albumin in PBS) for 1 h at RT. For immunolabeling, embryos were incubated with a rabbit anti-H3K9 me3 antibody (Abcam, Cambridge, MA, USA) or a mouse anti-alpha tubulin (acetyl K40) antibody (Abcam, Cambridge, MA, USA) or a mouse anti-CDX2 antibody (BioGenex, Fremont, California, USA) overnight at 4°C. On the second day, according to the species of primary antibody raised, the embryos were washed three times in PBS and incubated with Alexa Fluor 555-labeled goat anti-rabbit IgG (Beyotime, Shanghai, China) and Alexa Fluor 488-labeled goat anti-mouse IgG (Beyotime, Shanghai, China) secondary antibodies, respectively, for 1 h at RT. Finally, the embryos were counterstained with DAPI (Sigma, St. Louis, MO, USA) for 2–3 min.

All samples were mounted on glass slides with a drop of Antifade Mounting Medium (Beyotime, Shanghai, China) for indirect immunofluorescence examination using a laser-

scanning confocal microscope (Zeiss, Jena, Germany). The mean density (integrated optical density sum/area sum) of fluorescence signals was measured using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD). The area of each nucleus was outlined manually, and the mean fluorescence intensity was measured for H3K9me3, and DAPI images. The mean fluorescence intensities were divided by the acquisition times of the corresponding signal. These corrected mean fluorescence intensities were multiplied by the nuclear areas to obtain the total fluorescence intensities for H3K9 me3 and DAPI. Finally, the levels of H3K9 me3 were divided by total DNA contents (DAPI) to calculate normalized H3K9me3 quantities.

Regarding Ac α-tubulin K40 levels measurement, the same protocol was performed. The embryos were at the same developmental stage and were photographed with same instrument parameters. The number of embryos in each group was 10-15. Regarding the ICM/TE staining, as CDX2 was specifically expressed in tropho ectoderm, its expression was not detectable in ICM. The ratio of ICM/TE was thus the cell number of ICM divided by the cell number of TE. The cell number of ICM was the total cell number minus the CDX2 positive cell number, and the cell number of TE was the CDX2 positive cell number (An, Peng et al. 2019).

Detection of apoptosis in blastocysts

Embryonic apoptosis staining was performed using DeadEnd^{TM} TUNEL (Promega, Madison, WI) as described previously(Zhang, Qu et al. 2018). Blastocysts (day 7, n=10-15) were fixed in 4% paraformaldehyde at RT for 2 h, and then permeabilized with 0.5% Triton X-100 for 5 min. After three washes, the blastocysts were transferred to 100 μL Ebuffer droplets at RT for 8 min. The embryos were then incubated in the apoptosis staining working solution (an Ebuffer: Nucleotide Mix: rTdT mixture at 45 μL: 5 μL: 1 μL) in the dark at RT for 1 h. After this, 2× SSC was used to treat embryos for 15 min, followed by an incubation in 25 μg/mL

RNase A-contained PBS and DAPI for 3 min. Subsequently, the embryos were washed with PBS-PVA for three times before mounting for observation under a Nikon Eclipse Ti-S fluorescence microscope (Nikon, Tokyo, Japan).

Embryo transfer

The embryo transfer was carried out as previously reported(Qu, Qing et al. 2017). Healthy cows (3-5 years old) were used as the SCNT embryo recipients. One or two cloned embryos at the blastocyst stage were loaded in one 0.25 mL straw. The straw containing embryos was incubated in HEPES-buffered TCM-199 supplemented with 10% FBS at 37℃ within 1h, and then transported from the laboratory to the experimental farm. The embryos were transferred nonsurgically into the uterine horn of the recipients on day 7 of their natural estrous cycle. Rectal palpation and ultrasonography were applied during the pregnancy.

Statistical analysis

All experiments were repeated at least three times. Statistical analysis was conducted using the SPSS software package (SPSS Inc., Chicago, IL, USA). The cleavage and blastocyst formation ratio was assessed at 24 h, 48 h, day 6 and 7 post SCNT, respectively. The ratio of pronuclei number, the ratio of cleavage formation, and the ratio of blastocyst formation were analyzed using the χ^2 test. The average cleavage time, the relative intensity of H3K9me3 and Ac α-tubulin K40, apoptotic index, total cell number, and ICM/TE index were analysed using one-way ANOVA. A difference with $P < 0.05$ was considered statistically significant.

Results

Evaluation of quality and quantity of isolated sperm small RNA

The ratio of OD (optical density) 260/280 of isolated small RNA was ranged between 1.8 and 2.0, and the concentration was 100–500 ng/μL. In the results of small RNA quantification in

RT-qPCR, no significant differences in the expression levels of miR-34c and miRNA-449b were observed in MII oocytes, parthenogenesis, and control SCNT embryos (C-NT). However, compared to MII oocytes, parthenogenesis, or C-NT, the expression levels of miR-34c and miRNA-449b were significantly higher in *in vitro* fertilized (IVF) zygotes (Fig.1A and Fig.1B). Considering the gene silencing prior to zygotic gene activation, the expression levels of sperm-borne miR-34c and miRNA-449b in the sperm small RNA in SCNT (T-NT) group were significantly higher than that from IVF zygote.

Sperm-borne small RNA promote the formation of two pronuclear-like structures in SCNT embryos

SCNT embryos with less than three pronuclei were regarded as normal pronuclear structures. As shown in the Fig. 1-C, the formation ratio of abnormal pronuclear structures was significantly higher in the C-NT group, compared to those in the T-NT and IVF groups ($P < 0.001$). These results indicate that sperm-born small RNA play a crucial role in the formation of normal pronuclear-like structures.

Sperm-borne small RNA impact the first cleavage time of SCNT embryos

Previous studies have reported that the first cleavage time is different between SCNT and IVF embryos (Isom, Li et al. 2012). We hypothesized that sperm-born small RNA was one of the reasons for the difference. To verify the hypothesis, we recorded the average cleavage time of the embryos in the IVF, C-NT, and T-NT groups. As can be seen in Fig. 1-D, the average cleavage time of each groups recorded were 26.5 h, 23.9 h and 25.7 h, respectively. The first cleavage of embryos in the C-NT group requires significantly less time than that in the IVF and T-NT groups ($P<0.001$). In particular, the cleavage time of the embryos in IVF and T-NT groups was significantly different ($P=0.008$). The results indicate that sperm-borne small RNA can delay the first cleavage time.

Sperm-borne small RNA significantly reduce H3K9me3 levels in SCNT embryos

To evaluate the impacts of sperm-borne small RNA on nuclear epigenetic reprogramming of early embryos, the level of H3K9me3, which was a crucial epigenetic obstacle to complete nuclear reprogramming of SCNT embryos, was compared among the embryos in the IVF, T-NT, and C-NT groups. As shown in Fig.2, the level of H3K9me3 was rarely observed in the female pronuclear of IVF zygote, whereas it was substantially observed in all pseudo-pronuclei during the pronuclear stage in SCNT embryos. Embryos in IVF group at the two-cell stage showed lower levels of H3K9me3 compared to that in the C-NT group(P<0.01). In addition, H3K9me3 levels at the pronuclear (P<0.001) and two-cell ($P<0.05$) stages in the T-NT group were significantly lower than those in the C-NT group. These results suggest that sperm small RNA involve in nuclear epigenetic reprogramming, indicated by H3K9me3 expression levels.

Sperm-borne small RNA enhance α-tubulin K40 acetylation during the first mitotic cell cycle in bovine SCNT embryos

To verify the hypothesis that the prolonged first cleavage time was associated with sperm-borne small RNA, the level of Ac α-tubulin K40 was assessed at different developmental stages during the first cleavage in bovine embryos. As seen in the Fig.3, the level of Ac α-tubulin K40 were observed significantly lower in the C-NT group than that in the IVF group at both anaphase (P<0.001) and telophase(P<0.01). After the sperm small RNA were injected into the reconstructed embryo, a high level of Ac α-tubulin K40 was observed, and it was significantly higher than that in the C-NT group at both anaphase (P<0.001) and telophase (P<0.05). These results show that sperm small RNA regulate the acetylation level of α-tubulin during the first embryonic cleavage.

Sperm-borne small RNA enhance pre-implantation development of SCNT embryos

To assess the effect of sperm-borne small RNA on the developmental potential of bovine SCNT embryos, the cleavage, blastocyst formation ratio, apoptosis index (day 7), and ICM/TE ratio (day 7) of blastocysts in IVF, T-NT and C-NT groups were detected. The cleavage ratio in the T-NT (P<0.01) or IVF (P<0.01) groups were substantially lower than that in the C-NT group at 24 h after activation or fertilization; and at 48 h, the cleavage ratio in the T-NT group (P<0.05) or IVF group (P<0.05) was significantly higher than that in the C-NT group (Fig. 4A and 4B). Although no significant differences were observed in the blastocyst formation ratio in all three groups on day 6 after activation, the blastocyst formation ratio on day 7 was significantly lower in C-NT group than those in the T-NT (P<0.01) and IVF (P<0.01) groups (Fig. 4C and 4D). In addition, the apoptosis index of blastocysts was significantly higher in the C-NT group than that in the T-NT (P<0.001) or IVF (P<0.001) groups (Fig. 5A and 5B). The ICM/TE ratio was significantly lower in the C-NT group than that in the T-NT (P<0.05) or IVF (P<0.05) groups (Fig. 5C and 5D). These findings suggest that sperm-borne small RNA significantly enhance the developmental competence of SCNT embryos.

Sperm-borne small RNA impact the developmental potential of cloned bovine embryos *in vivo* and cloned offspring

To investigate the effects of sperm-borne small RNA on the developmental competence of SCNT embryos *in vivo*, SCNT embryos at blastocyst stage from T-NT and C-NT groups were transferred to synchronized recipient cows. The pregnancy rate on 40, 60, 90, and 120 day post embryo transfer (PET) was recorded and compared. As shown in Fig. 6A1, no significant difference was detected in the pregnancy rate on day 40 PET between these two groups (P=0.979). However, pregnancy rates on day 60 (P=0.022), 90 (P=0.005) and 120 (P=0.006) were significantly higher in T-NT group than that in the C-NT group (Fig. 6A1). In

the T-NT group, 16 calves were born from 82 recipients, while in the C-NT group, 6 calves were born from 85 recipients. The birth rate of the T-NT group was significantly higher than that in the C-NT group (P=0.032) (Fig. 6A2). Moreover, as shown Fig. 6A3, the birth weight of calves in the T-NT group (48.7±0.9kg) was significantly smaller than that in the C-NT group (54.8±0.7 kg) (P=0.007). Among 16 calves in the T-NT group, 5 calves died after birth, and 11 calves were alive (Fig. 6A4). While in the C-NT group, 4 of 6 calves were died shortly after birth (Fig. 6A4). These results indicate that sperm-borne small RNA have obviously positive effects on the *in vivo* developmental competence of SCNT embryos in bovine PET. Moreover, they can significantly alleviate the developmental abnormality of cloned offspring particularly birth weight.

Discussion

Small RNA is important in spermatogenesis, developmental regulation, as well as functions of Sertoli and Leydig cells in structural and nutritional support to germ cells (Hilz, Modzelewski et al. 2016). Down-regulation of miRNA-188-3p is associated with the apoptosis of spermatogenic cells (Song, Meng et al. 2017). The transgenerational response to paternal stress is regulated by small sperm-borne non-coding RNAs, specifically small RNA (Short, Yeshurun et al. 2017). Sperm-borne small RNA may change the embryonic molecular makeup post-fertilization, thereby altering its growth trajectory and ultimately affecting the phenotype of the adult offspring (Fullston, Ohlsson-Teague et al. 2016). Numerous studies have confirmed that sperm-borne small RNA is important during the early embryonic development(Liu, Pang et al. 2012, Yuan, Schuster et al. 2016, Guo, Chao et al. 2017). Yuan *et al.* reported that paternal miR-34c is important for the first cleavage (Yuan, Schuster et al. 2016). Our previous studies showed that sperm-borne miRNA-449b improves the epigenetic reprograming and decreases the apoptosis index of early embryos (Wang, Gao et al. 2017). Guo *et al.* found that the body weights of F1 mice produced from RNA-deficient sperm were

lower than those of controls (Guo, Chao et al. 2017). Thus, we hypothesized that some sperm-borne factors are associated with abnormal development of SCNT embryos. To verify this hypothesis, we injected sperm small RNA into cloned embryos and investigated their effects on embryonic development. The results suggest that sperm small RNA actually have an effect on developmental competence of SCNT embryo in both *in vitro* and *in vivo* studies.

As described earlier, abnormal epigenetic reprograming of donor nuclei after SCNT, such as DNA methylation and histone modification, was thought to be the major cause of low efficiency. These epigenetic markers on chromatin were thought to be at the core of genome reprograming after fertilization or SCNT (Zhao, Whyte et al. 2010). To clarify the underlying mechanisms, we focused on the expression level of H3K9me3, which is associated with the repression of chromatin, and the formation of heterochromatin (Pan, He et al. 2015). Our data showed that H3K9me3 was detected only in female pronuclei during the pronuclear period in IVF embryos. At the two-cell stage, H3K9me3 was detected in the nuclei of all three groups of embryos. We also observed that the level of H3K9me3 in the C-NT group was significantly higher than that in the IVF group at the 2PN and two-cell stages. This finding is consistent with the previous finding in cloned bovine embryos (Pan, He et al. 2015), indicating that abnormal H3K9me3 reprogramming exists in almost all SCNT embryos. In this study, H3K9me3 levels were significantly reduced after the injection of sperm-borne small RNA, suggesting that sperm-borne small RNA contribute to the reduction of NT-mediated aberrant nuclear reprograming in cloned bovine embryos. Our previous study reported miRNA-125b is a key epigenetic regulatory factor that promotes nuclear transfer reprogramming via targeting SUV39H1, to down-regulate H3K9me3 (Zhang, Qu et al. 2017). In another study, we used deep sequencing to analyse small RNA in bovine sperm, and we found miRNA-125b was rich in bovine sperm (Du, Wang et al. 2014). We conjecture miRNA-125b and other sperm-borne small RNA might regulate H3K9me3 by targeting the

genes involved in the associated pathway.

Abnormal reprograming causes abnormal cleavage, resulting in differences in the start time of first cleavage between NT and IVF embryos (Liu, Pang et al. 2012, Fullston, Ohlsson-Teague et al. 2016). Numerous studies have indicated that the first cleavage time is an indicative of the embryonic development (Isom, Li et al. 2012, Milazzotto, Goissis et al. 2016, Ochota and Nizanski 2017). Therefore, the identifications of the factors associated with the first cleavage time are crucial in the study of early embryonic development. In our study, we observed that the first cleavage time was reduced in SCNT embryos compared to that of IVF embryos. The timing of first cleavage in the T-NT group was significantly delayed than that in the C-NT group. Meanwhile, the cleavage ratio at 48 h in the experimental group was higher than that in the C-NT group. These data suggest that sperm-borne small RNA can regulate both the first cleavage time and early embryo development.

Sperm were previously found to be involved in the formation of zygote centrosomes and the assembly of microtubules during the first mitosis (Schatten, Schatten et al. 1986, Sutovsky and Schatten 2000, Kwon, Lee et al. 2010). Centrosome complexes play an important role in the process of embryonic development. Through their ability to organize microtubules, centrosomes can promote cell polarity and cell division and also decide cell fates (Schatten and Sun 2014). During the first cell cycle in SCNT embryos, abnormal centrosomes and failed “pronuclear” migration are related to errors in spindle morphology, chromosome alignment, and cytokinesis, which can lead to chromosome instability and failure (Dai, Wang et al. 2006). Chromatin is a scaffold structure for genomic DNA and a dynamic entity that is linked to transcriptional regulation, DNA replication, and DNA repair(Berger 2007). Embryonic development reduction is mainly caused by the abnormal functioning of cytoplasmic factors involved in cytokinesis(Yamada and Egli 2017). These studies indicate that such abnormal cytokinesis may occur in the reconstructed embryos

derived from nuclear transfer without sperm. Meanwhile, abnormal cytokinesis may be the main reason for the difference in first cleavage time difference between SCNT and IVF embryos. Microtubules are cytoskeletal components and play an important role in cytokinesis. α-Tubulin is a major component of microtubules that contributes to the formation of spindles and midbodies (Schatten, Simerly et al. 1988). Ac α-Tubulin K40 is one of the most important post-translational modifications of microtubules(Schatten, Simerly et al. 1988, Ly, Elkhatib et al. 2016). Studies have shown that acetylation affects the stability of microtubules, and an increase in acetylated α-tubulin levels can enhance the stability of mitotic microtubules (Miao, Zhou et al. 2017, Steinhauser, Kloble et al. 2017). We hypothesized that the sperm-borne small RNA may regulate cytoskeleton function through the Ac α-tubulin K40, thus affecting microtubule assembly, cytokinesis, and chromosome segregation, and further affecting the embryonic first cleavage. The first embryonic cell cycle ends after zygote chromosome separation during the first mitosis (Hou, Ma et al. 2014). In our study, we measured the levels of Ac α-tubulin K40 in IVF and SCNT embryos before first cleavage was completed. We found that sperm-borne small RNA plays a crucial role in bovine embryo mitotic progression by regulating cytoskeleton modification. Our data also demonstrate that sperm-borne small RNA can enhance the level of α-tubulin K40 acetylation in SCNT embryos at anaphase and telophase during first cleavage. HDAC6, as an important regulator, was predicted to be associate with acetylated α-tubulin (Schatten and Sun 2014). We previously found that HDAC6 was regulated by some small RNAs which are abundantly expressed in bovine sperm (Du, Wang et al. 2014). We hypothesized that sperm-borne small RNA regulates α-tubulin acetylation by targeting genes involving this pathway. Therefore, further studies will be required to determine how sperm-borne small RNA regulates Ac α-tubulin K40 during the first mitotic division in bovine embryos.

The cleavage and blastocyst formation ratio between SCNT and IVF groups was observed in this study. The results are consistent with our previous study and others reports (Wang, Gao et al. 2017, Zhang, Qu et al. 2017). More parameters, including the apoptotic index and ICM/TE ratio, were carried out to comprehensively assess the developmental competence of SCNT embryos (Qu, Qing et al. 2017). According to all these parameters, we could conclude that sperm-borne small RNA supplementation can significantly enhance the developmental competence of SCNT *in vitro*. Pregnancy rate and offspring healthy status are criteria for the developmental competence of SCNT embryos. Therefore, to assess the effect of sperm-borne small RNA on the *in vivo* developmental competence of SCNT embryos, embryo transfer experiment was conducted. Compared with normal fertilized fetal calves, most of the cloned offspring showed various abnormalities, such as high loss rate throughout all pregnancy during, placental overgrowth, increased body weight, immuno-system anomalies, and a short life span (Matoba and Zhang 2018). In addition, most of the cloned offspring manifested LOS which was reported to be related to mis-regulated imprinted genes (Smith, Suzuki et al. 2012). Previous studies found abnormal expression of MHC and PAG2 were responsible abnormal placenta development (Davies, Hill et al. 2004, Whitworth, Spate et al. 2010). Our previous research found XIST, PEG3, IGF2, IGF2R, and H19 were abnormally expressed in LOS calves and DNA methylation of XIST, H19 and IGF2R was abnormal in the placenta of LOS. Also, miRNAs profiles in cloned offspring manifested different from those of fertilized offspring (Su, Wang et al. 2014, Su, Liu et al. 2015). In our subsequent studies, we performed high-throughput sequencing of sperm miRNA, and found that the target genes of sperm miRNAs were associated with placental development and imprinted genes (Du, Wang et al. 2014). In this study, the treated sperm-borne small RNA group showed higher pregnancy rates than the control group on day 60, day 90, and day 120. In the T-NT group, the birth weight was significantly lower than that in the C-NT group. The

results indicate that sperm-borne small RNA plays an important role on SCNT embryo development *in vivo*, decrease the abnormal offspring and improve cloning efficiency.

To conclude, our study demonstrates that sperm-borne small RNA can (1) affect both histone modification and the nuclear remodeling of early embryos by reducing the level of H3K9 me3 and (2) up regulate the levels of Ac α-tubulin K40 during cytokinesis and slow the time of first cleavage and (3) benefit the development of cloned embryo both *in vitro* and *in vivo* (Fig. 6D). Further investigation on the effects of sperm-borne small RNA is anticipated to provide new ideas elucidating the developmental mechanisms of early embryos, with an aim of improving the efficiency of SCNT.

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Authors' contributions

Yong Zhang, Suzhu Qing, and Yongsheng Wang designed research; Pengxiang Qu, Zhenzi Zuo, and Ying Zhang performed research; Xiaonan Ma, Fang Qiao, Mengyun Wang and Yue Du analyzed data; Yongsheng Wang and Pengxiang Qu wrote the report; Pengxiang Qu, Yue Du, Zhengqing Liu, and Zhihan Niu revised the manuscript. All authors declare that they have no conflict of interest.

References

Akagi, S., M. Geshi and T. Nagai. Recent progress in bovine somatic cell nuclear transfer. Anim Sci J. 2013; **3**: 191-199.

Amanai, M., M. Brahmajosyula and A. C. Perry. A restricted role for sperm-borne microRNAs in mammalian fertilization. Biol Reprod. 2006; **6**: 877-884.

An, Q., W. Peng, Y. Cheng, Z. Lu, C. Zhou, Y. Zhang and J. Su. Melatonin supplementation during in vitro maturation of oocyte enhances subsequent development of bovine cloned embryos. J Cell Physiol. 2019.

Berger, S. L. The complex language of chromatin regulation during transcription. Nature. 2007; **7143**: 407-412.

Betthauser, J. M., M. Pfister-Genskow, H. Xu, P. J. Golueke, J. C. Lacson, R. W. Koppang, C. Myers, B. Liu, I. Hoeschele, K. J. Eilertsen and G. H. Leno. Nucleoplasmin facilitates reprogramming and in vivo development of bovine nuclear transfer embryos. Mol Reprod Dev. 2006; **8**: 977-986.

Chakrabarty, S., A. Das, A. Bhattacharya and G. Chakrabarti. Theaflavins depolymerize microtubule network through tubulin binding and cause apoptosis of cervical carcinoma HeLa cells. J Agric Food Chem. 2011; **5**: 2040-2048.

Chung, Y. G., S. Matoba, Y. Liu, J. H. Eum, F. Lu, W. Jiang, J. E. Lee, V. Sepilian, K. Y. Cha, D. R. Lee and Y. Zhang. Histone Demethylase Expression Enhances Human Somatic Cell Nuclear Transfer Efficiency and Promotes Derivation of Pluripotent Stem Cells. Cell Stem Cell. 2015; **6**: 758-766.

Dai, Y., L. Wang, H. Wang, Y. Liu, N. Li, Q. Lyu, D. L. Keefe, D. F. Albertini and L. Liu. Fate of centrosomes following somatic cell nuclear transfer (SCNT) in bovine oocytes. Reproduction. 2006; **6**: 1051-1061.

Davies, C. J., J. R. Hill, J. L. Edwards, F. N. Schrick, P. J. Fisher, J. A. Eldridge and D. H. Schlafer. Major histocompatibility antigen expression on the bovine placenta: its relationship to abnormal pregnancies and retained placenta. Anim Reprod Sci. 2004: 267-280.

Du, Y., X. G. Wang, B. Wang, W. J. Chen, R. J. He, L. Zhang, X. P. Xing, J. M. Su, Y. S. Wang and

Y. Zhang. Deep Sequencing Analysis of microRNAs in Bovine Sperm. Molecular Reproduction and Development. 2014; **11**: 1042-1052.

Fullston, T., E. M. Ohlsson-Teague, C. G. Print, L. Y. Sandeman and M. Lane. Sperm microRNA Content Is Altered in a Mouse Model of Male Obesity, but the Same Suite of microRNAs Are Not Altered in Offspring's Sperm. PLoS One. 2016; **11**: e0166076.

Gao, L. R., S. Li, J. Zhang, C. Liang, E. N. Chen, S. Y. Zhang, M. Chuai, Y. P. Bao, G. Wang and X. Yang. Excess Imidacloprid Exposure Causes the Heart Tube Malformation of Chick Embryos. J Agric Food Chem. 2016; **47**: 9078-9088.

Guo, L., S. B. Chao, L. Xiao, Z. B. Wang, T. G. Meng, Y. Y. Li, Z. M. Han, Y. C. Ouyang, Y. Hou, Q. Y. Sun and X. H. Ou. Sperm-carried RNAs play critical roles in mouse embryonic development. Oncotarget. 2017; **40**: 67394-67405.

Hilz, S., A. J. Modzelewski, P. E. Cohen and A. Grimson. The roles of microRNAs and siRNAs in mammalian spermatogenesis. Development. 2016; **17**: 3061-3073.

Hou, L., F. Ma, J. Yang, H. Riaz, Y. Wang, W. Wu, X. Xia, Z. Ma, Y. Zhou, L. Zhang, W. Ying, D. Xu, B. Zuo, Z. Ren and Y. Xiong. Effects of histone deacetylase inhibitor oxamflatin on in vitro porcine somatic cell nuclear transfer embryos. Cell Reprogram. 2014; **4**: 253-265.

Isom, S. C., R. F. Li, K. M. Whitworth and R. S. Prather. Timing of first embryonic cleavage is a positive indicator of the in vitro developmental potential of porcine embryos derived from in vitro fertilization, somatic cell nuclear transfer and parthenogenesis. Mol Reprod Dev. 2012; **3**: 197-207.

Jodar, M., S. Selvaraju, E. Sendler, M. P. Diamond, S. A. Krawetz and N. Reproductive Medicine. The presence, role and clinical use of spermatozoal RNAs. Hum Reprod Update. 2013; **6**: 604-624.

Krawetz, S. A. Paternal contribution: new insights and future challenges. Nat Rev Genet. 2005; **8**: 633-642.

Kwon, D. J., Y. M. Lee, I. S. Hwang, C. K. Park, B. K. Yang and H. T. Cheong. Microtubule distribution in somatic cell nuclear transfer bovine embryos following control of nuclear remodeling type. J Vet Sci. 2010; **2**: 93-101.

Liu, W. M., R. T. Pang, P. C. Chiu, B. P. Wong, K. Lao, K. F. Lee and W. S. Yeung. Sperm-borne microRNA-34c is required for the first cleavage division in mouse. Proc Natl Acad Sci U S A. 2012; **2**: 490-494.

Lu, Z., C. Zhang, C. Han, Q. An, Y. Cheng, Y. Chen, R. Meng, Y. Zhang and J. Su. Plasticizer bis(2-ethylhexyl) phthalate causes meiosis defects and decreases fertilization ability of mouse oocytes in vivo. J Agric Food Chem. 2019.

Ly, N., N. Elkhatib, E. Bresteau, O. Pietrement, M. Khaled, M. M. Magiera, C. Janke, E. Le Cam, A. D. Rutenberg and G. Montagnac. alphaTAT1 controls longitudinal spreading of acetylation marks from open microtubules extremities. Sci Rep. 2016: 35624.

Ma, X., Y. Zhang, F. Qiao, M. Wang, Z. Zuo, Y. Zhang and Y. Wang. Comparison of RNA extraction and microRNA detection protocols for a small amount of germinal vesicle oocytes in bovine. Anim Reprod Sci. 2018.

Maki, N., R. Suetsugu-Maki, S. Sano, K. Nakamura, O. Nishimura, H. Tarui, K. Del Rio-Tsonis, K. Ohsumi, K. Agata and P. A. Tsonis. Oocyte-type linker histone B4 is required for transdifferentiation of somatic cells in vivo. FASEB J. 2010; **9**: 3462-3467.

Matoba, S., Y. Liu, F. Lu, K. A. Iwabuchi, L. Shen, A. Inoue and Y. Zhang. Embryonic development following somatic cell nuclear transfer impeded by persisting histone methylation. Cell. 2014; **4**: 884-895.

Matoba, S. and Y. Zhang. Somatic Cell Nuclear Transfer Reprogramming: Mechanisms and Applications. Cell Stem Cell. 2018; **4**: 471-485.

Matsubara, K., A. R. Lee, S. Kishigami, A. Ito, K. Matsumoto, H. Chi, N. Nishino, M. Yoshida and Y. Hosoi. Dynamics and regulation of lysine-acetylation during one-cell stage mouse embryos. Biochem Biophys Res Commun. 2013; **1**: 1-7.

Miao, Y., C. Zhou, Z. Cui, L. Tang, X. ShiYang, Y. Lu, M. Zhang, X. Dai and B. Xiong. Dynein promotes porcine oocyte meiotic progression by maintaining cytoskeletal structures and cortical granule arrangement. Cell Cycle. 2017; **21**: 2139-2145.

Milazzotto, M. P., M. D. Goissis, J. L. Chitwood, K. Annes, C. A. Soares, J. Ispada, M. E. Assumpcao and P. J. Ross. Early cleavages influence the molecular and the metabolic pattern

of individually cultured bovine blastocysts. Mol Reprod Dev. 2016; **4**: 324-336.

Nakagawa, U., D. Suzuki, M. Ishikawa, H. Sato, K. Kamemura and A. Imamura. Acetylation of alpha-tubulin on Lys40 is a widespread post-translational modification in angiosperms. Biosci Biotechnol Biochem. 2013; **7**: 1602-1605.

Ochota, M. and W. Nizanski. Time of early cleavage affects the developmental potential of feline preimplantation embryos in vitro. Theriogenology. 2017: 26-31.

Ostermeier, G. C., D. Miller, J. D. Huntriss, M. P. Diamond and S. A. Krawetz. Reproductive biology: delivering spermatozoan RNA to the oocyte. Nature. 2004; **6988**: 154.

Pan, Y., H. He, Y. Cui, A. R. Baloch, Q. Li, J. Fan, J. He and S. Yu. Recombinant Human Bone Morphogenetic Protein 6 Enhances Oocyte Reprogramming Potential and Subsequent Development of the Cloned Yak Embryos. Cell Reprogram. 2015; **6**: 484-493.

Peng, H., J. Shi, Y. Zhang, H. Zhang, S. Liao, W. Li, L. Lei, C. Han, L. Ning, Y. Cao, Q. Zhou, Q. Chen and E. Duan. A novel class of tRNA-derived small RNAs extremely enriched in mature mouse sperm. Cell Res. 2012; **11**: 1609-1612.

Qu, P., S. Qing, R. Liu, H. Qin, W. Wang, F. Qiao, H. Ge, J. Liu, Y. Zhang, W. Cui and Y. Wang. Effects of embryo-derived exosomes on the development of bovine cloned embryos. PLoS One. 2017; **3**: e0174535.

Qu, P., Y. Zhao, R. Wang, Y. Zhang, L. Li, J. Fan and E. Liu. Extracellular vesicles derived from donor oviduct fluid improved birth rates after embryo transfer in mice. Reprod Fertil Dev. 2018.

Rassoulzadegan, M., V. Grandjean, P. Gounon, S. Vincent, I. Gillot and F. Cuzin. RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. Nature. 2006; **7092**: 469-474.

Sato, F., S. Tsuchiya, S. J. Meltzer and K. Shimizu. MicroRNAs and epigenetics. FEBS J. 2011; **10**: 1598-1609.

Schatten, G., C. Simerly, D. J. Asai, E. Szoke, P. Cooke and H. Schatten. Acetylated alpha-tubulin in microtubules during mouse fertilization and early development. Dev Biol. 1988; **1**: 74-86.

Schatten, H., G. Schatten, D. Mazia, R. Balczon and C. Simerly. Behavior of centrosomes during fertilization and cell division in mouse oocytes and in sea urchin eggs. Proc Natl Acad Sci U S A. 1986; **1**: 105-109.

Schatten, H. and Q. Y. Sun. Posttranslationally modified tubulins and other cytoskeletal proteins: their role in gametogenesis, oocyte maturation, fertilization and Pre-implantation embryo development. Adv Exp Med Biol. 2014: 57-87.

Shin, M. R., S. W. Park, H. Shim and N. H. Kim. Nuclear and microtubule reorganization in nuclear-transferred bovine embryos. Mol Reprod Dev. 2002; **1**: 74-82.

Short, A. K., S. Yeshurun, R. Powell, V. M. Perreau, A. Fox, J. H. Kim, T. Y. Pang and A. J. Hannan. Exercise alters mouse sperm small noncoding RNAs and induces a transgenerational modification of male offspring conditioned fear and anxiety. Transl Psychiatry. 2017; **5**: e1114.

Smith, L. C., J. Suzuki, Jr., A. K. Goff, F. Filion, J. Therrien, B. D. Murphy, H. R. Kohan-Ghadr, R. Lefebvre, A. C. Brisville, S. Buczinski, G. Fecteau, F. Perecin and F. V. Meirelles. Developmental and epigenetic anomalies in cloned cattle. Reprod Domest Anim. 2012: 107-114.

Song, R., G. W. Hennig, Q. Wu, C. Jose, H. Zheng and W. Yan. Male germ cells express abundant endogenous siRNAs. Proc Natl Acad Sci U S A. 2011; **32**: 13159-13164.

Song, W. Y., H. Meng, X. G. Wang, H. X. Jin, G. D. Yao, S. L. Shi, L. Wu, X. Y. Zhang and Y. P. Sun. Reduced microRNA-188-3p expression contributes to apoptosis of spermatogenic cells in patients with azoospermia. Cell Prolif. 2017; **1**.

Steinhauser, K., P. Kloble, N. N. Kreis, A. Ritter, A. Friemel, S. Roth, J. M. Reichel, J. Michaelis, M. A. Rieger, F. Louwen, F. Oswald and J. Yuan. Deficiency of RITA results in multiple mitotic defects by affecting microtubule dynamics. Oncogene. 2017; **15**: 2146-2159.

Su, J., X. Liu, H. Sun, Y. Wang, Y. Wu, Z. Guo and Y. Zhang. Identification of differentially expressed microRNAs in placentas of cloned and normally produced calves by Solexa sequencing. Anim Reprod Sci. 2015: 64-74.

Su, J., Y. Wang, X. Xing, J. Liu and Y. Zhang. Genome-wide analysis of DNA methylation in bovine placentas. BMC Genomics. 2014: 12.

Sutovsky, P. and G. Schatten. Paternal contributions to the mammalian zygote: fertilization after sperm-egg fusion. Int Rev Cytol. 2000: 1-65.

Tao, J., Y. Zhang, X. Zuo, R. Hong, H. Li, X. Liu, W. Huang, Z. Cao and Y. Zhang. DOT1L inhibitor improves early development of porcine somatic cell nuclear transfer embryos. PLoS One. 2017; **6**: e0179436.

Wang, B., Y. Wang, M. Zhang, Y. Du, Y. Zhang, X. Xing, L. Zhang, J. Su, Y. Zhang and Y. Zheng. MicroRNA-34c expression in donor cells influences the early development of somatic cell nuclear transfer bovine embryos. Cell Reprogram. 2014; **6**: 418-427.

Wang, M., Y. Gao, P. Qu, S. Qing, F. Qiao, Y. Zhang, J. Mager and Y. Wang. Sperm-borne miR-449b influences cleavage, epigenetic reprogramming and apoptosis of SCNT embryos in bovine. Sci Rep. 2017; **1**: 13403.

Wang, Y. S., S. Tang, Z. X. An, W. Z. Li, J. Liu, F. S. Quan, S. Hua and Y. Zhang. Effect of mSOF and G1.1/G2.2 media on the developmental competence of SCNT-derived bovine embryos. Reprod Domest Anim. 2011; **3**: 404-409.

Whitworth, K. M., L. D. Spate, R. Li, A. Rieke, P. Sutovsky, J. A. Green and R. S. Prather. Activation method does not alter abnormal placental gene expression and development in cloned pigs. Mol Reprod Dev. 2010; **12**: 1016-1030.

Yamada, M. and D. Egli. Genome Transfer Prevents Fragmentation and Restores Developmental Potential of Developmentally Compromised Postovulatory Aged Mouse Oocytes. Stem Cell Reports. 2017; **3**: 576-588.

Yan, W., K. Morozumi, J. Zhang, S. Ro, C. Park and R. Yanagimachi. Birth of mice after intracytoplasmic injection of single purified sperm nuclei and detection of messenger RNAs and MicroRNAs in the sperm nuclei. Biol Reprod. 2008; **5**: 896-902.

Yan, Z. H., Y. Y. Zhou, J. Fu, F. Jiao, L. W. Zhao, P. F. Guan, S. Z. Huang, Y. T. Zeng and F. Zeng. Donor-host mitochondrial compatibility improves efficiency of bovine somatic cell nuclear transfer. BMC Dev Biol. 2010: 31.

Yuan, S., A. Schuster, C. Tang, T. Yu, N. Ortogero, J. Bao, H. Zheng and W. Yan. Sperm-borne miRNAs and endo-siRNAs are important for fertilization and preimplantation embryonic

development. Development. 2016; **4**: 635-647.

Zhang, J., P. Qu, C. Zhou, X. Liu, X. Ma, M. Wang, Y. Wang, J. Su, J. Liu and Y. Zhang. MicroRNA-125b is a key epigenetic regulatory factor that promotes nuclear transfer reprogramming. J Biol Chem. 2017; **38**: 15916-15926.

Zhang, Y., P. Qu, X. Ma, F. Qiao, Y. Ma, S. Qing, Y. Zhang, Y. Wang and W. Cui. Tauroursodeoxycholic acid (TUDCA) alleviates endoplasmic reticulum stress of nuclear donor cells under serum starvation. PLoS One. 2018; **5**: e0196785.

Zhao, J., J. Whyte and R. S. Prather. Effect of epigenetic regulation during swine embryogenesis and on cloning by nuclear transfer. Cell Tissue Res. 2010; **1**: 13-21.

Zhu, Z. Y., M. X. Jiang, L. Y. Yan, J. C. Huang, Z. L. Lei, Y. Jiang, Y. C. Ouyang, H. X. Zhang, Q. Y. Sun and D. Y. Chen. Cytoskeletal and nuclear organization in mouse embryos derived from nuclear transfer and ICSI: a comparison of agamogony and syngamy before and during the first cell cycle. Mol Reprod Dev. 2007; **5**: 655-663.

Figure Legends

Fig.1. Relative expression levels of miR-34c and miR-449b, the ratio of pronuclear structures, and first cleavage time in each group (A) Quantitative (q) PCR analysis of relative expression levels of miR-34c in MII oocytes, PA embryos, C-NT embryos, IVF embryos, and T-NT embryos. (B) qPCR analysis of relative expression levels of miR-449b. (C) The ratio of abnormal pronuclear structures in IVF, C-NT and T-NT group. (D) The first cleavage time of embryos in IVF, C-NT and T-NT group. MII, metaphase II; PA, parthenogenesis; C-NT, somatic cell nuclear transfer; T-NT, somatic cell nuclear transfer injected with sperm small RNA.

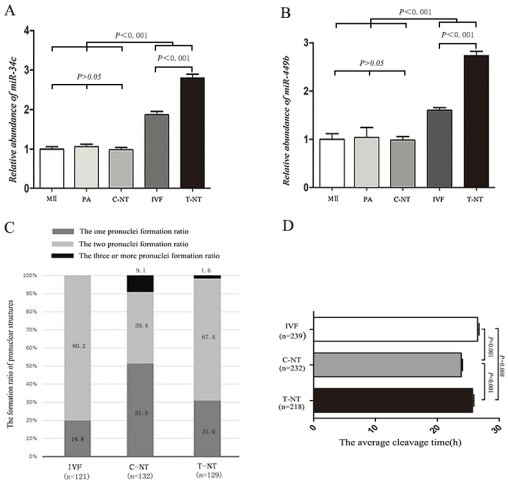

Fig.2. The level of H3K9me3 in IVF and SCNT embryos at pronuclear stage and 2-cell stage

(A) The staining pattern of H3K9me3 in IVF and SCNT (C-NT, T-NT) embryos at the pronuclear stage. H3K9me3: red; DNA: blue. Bar = 40 μm. Arrows indicate polar bodies. (B) Relative fluorescence intensities of H3K9me3 at the pronuclear stage. (C) The staining pattern of H3K9me3 in IVF and SCNT (C-NT, T-NT) embryos at 2-cell stage. H3K9me3: red; DNA: blue. Bar = 40 μm. Arrows indicate polar bodies. (D) Relative fluorescence intensities of H3K9me3 at 2-cell stage. H3K9me3, trimethylation of histone 3 lysine 9; SCNT, somatic cell nuclear transfer.

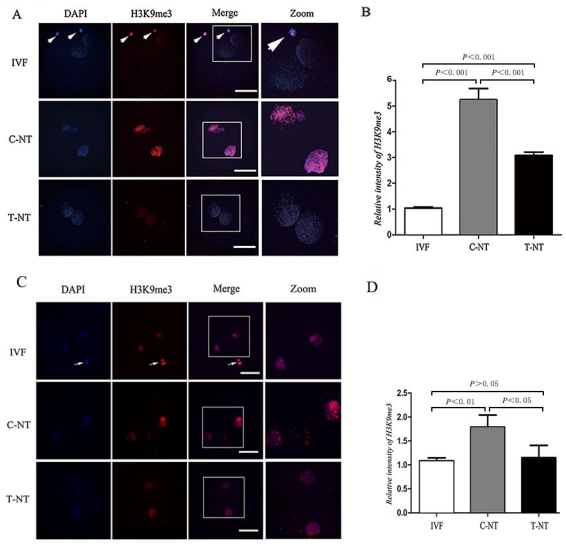

Fig.3. The level of α-tubulin K40 acetylation in bovine IVF and SCNT embryos at anaphase and telophase

(A) Representative photographs of α-tubulin K40 acetylation in bovine IVF and SCNT embryos at anaphase stage, α-Tubulin K40 acetylation: green; DNA: blue. Bar = 40 μm. Arrows indicate polar bodies. (B) The relative fluorescence intensity of α-tubulin K40 acetylation at anaphase. (C) Representative photographs of α-tubulin K40 acetylation in bovine IVF and SCNT embryos at telophase, α-Tubulin K40 acetylation: green; DNA: blue. Bar = 40 μm. Arrows indicate polar bodies. (D) The relative fluorescence intensity of α-tubulin K40 acetylation at telophase.

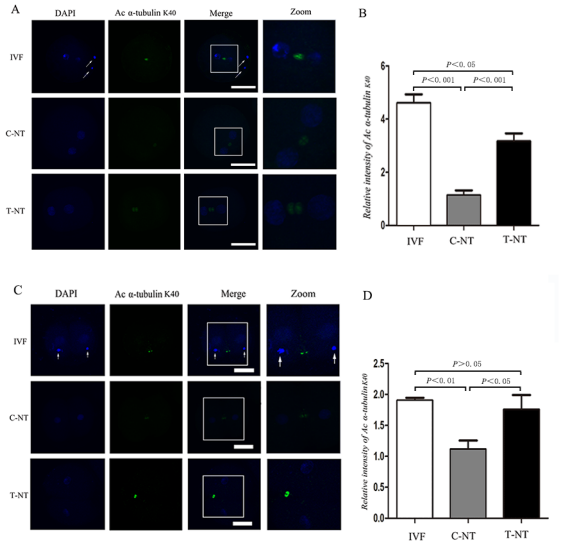

Fig.4. Cleavage ratios at 24h, 48h, and blastocyst ratios on day 6 and day 7 in IVF, C-NT, and T-NT groups.

(A) Representative photographs of embryos at 24h and 48h. Bar = 100 μm. (B) Cleavage ratio at 24 h (left) and 48h (right) derived from IVF, C-NT, and T-NT groups. (C) Representative photographs of embryos on day 6 and day 7. Bar = 100 μm. (D) Blastocyst ratios on day 6 (left) and day 7 (right).

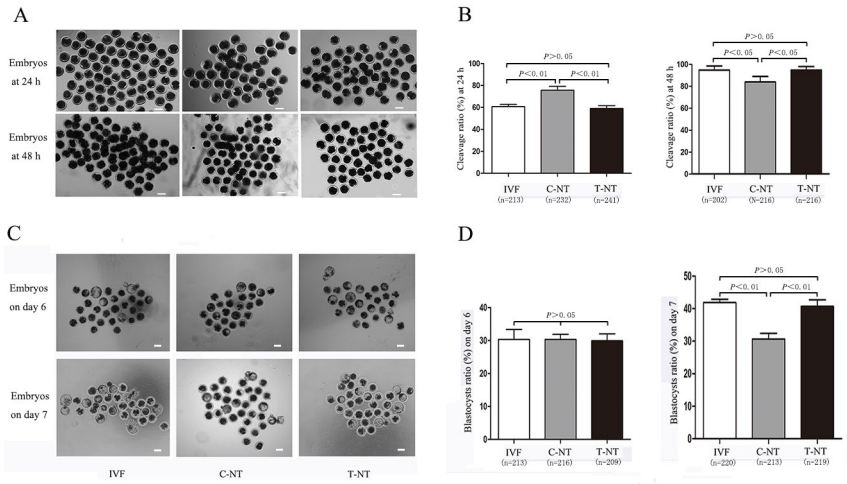

Fig.5. Apoptotic index and ICM/TE index in day 7 blastocyst in IVF, C-NT, and T-NT groups

The apoptotic blastomeres were detected by TUNEL (green). DNA was stained by DAPI (blue) to visualize all blastomeres. Bar = 40 μm. (B) Apoptotic index in blastocysts of IVF, C-NT, and T-NT groups. (C) TE were detected by Cdx2 antibody, a special marker of TE (green), DNA was stained by DAPI (blue) to visualize all blastomeres. Bar = 40 μm. (D) ICM/TE index in blastocysts of IVF, C-NT, and T-NT groups. ICM/TE, inner cell mass/tropho ectoderm.

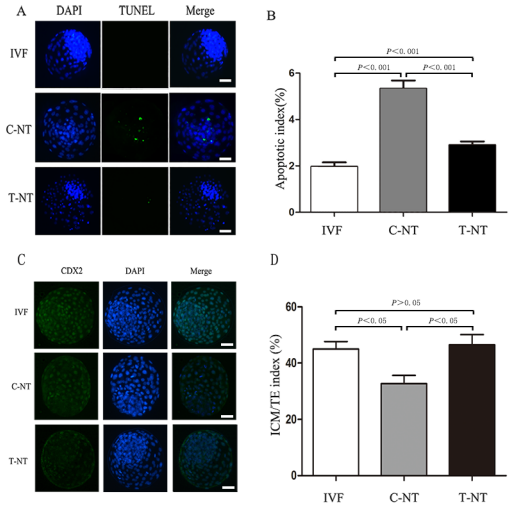

Fig.6. Pregnancy rates, birth rates, birth weight, and status of clonde calves in C-NT, and T-NT groups and graph of sperm-borne small RNA on cloning efficiency.

(A) Pregnancy rates (A1), birth rates (A2), birth weight (A3), and status (A4) of cloned calves in C-NT, and T-NT groups. (B) Representative photograph of calves shortly after birth. The calf marked * died shortly after birth and the calf marked △ was live after birth. (C) Cloned calf which was live at 1 week after birth. (D) Cartoon of sperm-borne small RNA on cloning efficiency injection of sperm-borne small RNA decreased the levels of H3K9 me3 and increased Ac